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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
G01N 33/543, 33/558, 33/58

A1

(11) International Publication Number: **WO 00/00826**

(43) International Publication Date: 6 January 2000 (06.01.00)

(21) International Application Number: PCT/GB99/02019

(22) International Filing Date: 28 June 1999 (28.06.99)

(30) Priority Data:
9814008.0 30 June 1998 (30.06.98) GB

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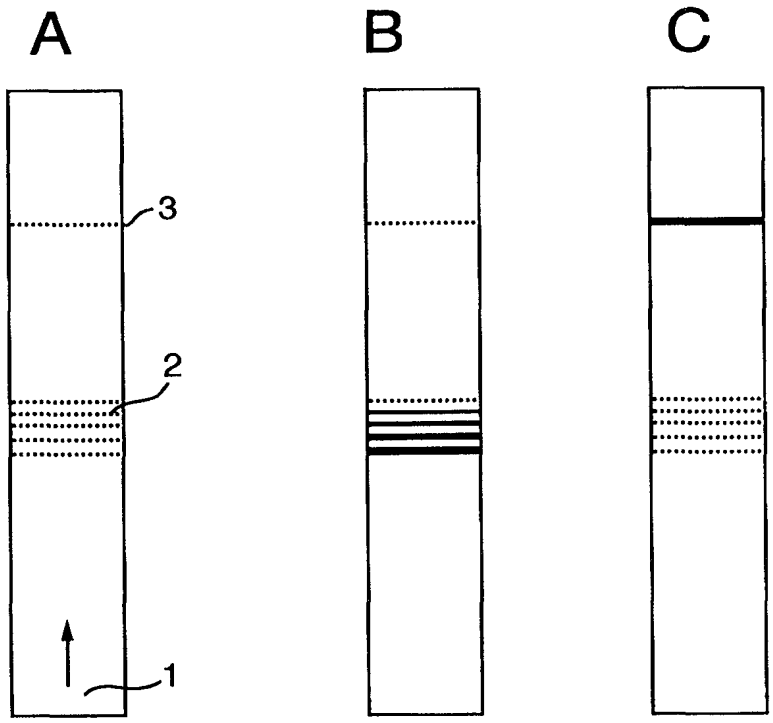
(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMMUNOMETRIC ASSAY

(57) Abstract

In a specific binding partner–mediated assay for an analyte, e.g. with a single binding site, a sample flows along a channel or permeable material from an application site 1. A labelled specific binding partner directed against the binding site on the analyte of interest is introduced in excess. At a site 2, a defined distance along the membrane or channel, there is immobilised a sufficient excess of the analyte to capture all of the labelled partner which has not previously bound the analyte of interest. The excess labelled partner thereby becomes immobilised, leaving labelled partner–analyte complex to continue to migrate along the material. At a further defined distance along the membrane or channel, i.e. at a "test site" 3, there is immobilised a second binding reagent with high affinity for the labelled partner–analyte conjugate, upon which this conjugate becomes immobilised and can be determined.



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IMMUNOMETRIC ASSAY

Field of the Invention

This invention relates to an immunometric assay whereby low concentrations of molecules having a single binding site can be rapidly detected and quantitated with minimal instrumentation by an untrained person.

Background of the Invention

Many immunological methods are available for the determination of haptens (small molecules) in aqueous solution. They suffer from two principal disadvantages, i.e. that as the concentration of hapten increases, the signal decreases; and that the signal is strongly affected by the concentration of the reagents used for its quantitation.

In an immunoassay, these disadvantages can be minimised or avoided, by using a sandwich wherein the analyte becomes attached to two different antibodies against distinct epitopes on the analyte. In the case of a small molecule, for example with a molecular weight below 1000, which displays only a single epitope, a sandwich cannot be formed.

Some proposals allow the quantitation of haptens using assays which obviate the disadvantages outlined above. US-A-5476770 describes a method wherein the hapten reacts immunologically with the antibody, bringing it into close proximity, so that a covalent bond may be created between the two molecules. The immunological bond is then broken, exposing the epitope on the hapten. Reaction with a labelled version of the same antibody then labels the hapten, the signal increasing for increasing amounts of the hapten. This method is technically demanding and time-consuming.

US-A-5236830 describes a method in which the hapten is derivatised before reaction, to introduce a marker; it is then removed from solution by reaction with an antibody attached to a carrier, and dissociated from that antibody; the marker concentration is assayed, giving a higher signal for higher analyte concentrations. The advantage of excess antibody concentration is also achieved, but the derivatisation and the dissociation can be time-consuming and are potential sources of error.

EP-A-0161107 describes a method of analysis of small molecules which employs a labelled antibody and an immobilised analogue of the analyte; the extent to which the immobilised analyte becomes labelled is greater for smaller amounts of analyte present in solution in the sample. This is recorded, to generate an inverse-sigmoid calibration

curve. While this method is described as an immunometric assay, on the ground of employing a labelled antibody rather than a labelled analogue of the hapten, neither a positive slope to the calibration curve nor the use of excess reagent is achieved.

US-A-5219730 describes the use of an idiotype-anti-idiotype immunoassay where analyte is detected by its inhibition of binding of a monoclonal antibody to the unoccupied binding site of a monoclonal antibody raised against the analyte. This requires the production of a pair of high-affinity antibodies for each analyte, which is time-consuming and difficult.

US-A-5641690 describes a variation of the foregoing method, whereby a hapten reacts with an antibody directed against the hapten; excess antibody then reacts with a secondary binding partner which may be an analogue of the hapten. A selective antibody is then introduced, which binds only to the antibody which has bound the hapten and not to the antibody which has bound to the secondary binding partner. This method requires the preparation of such a selective antibody, exhibiting a large difference between its affinities for the antibody-hapten complex and for the antibody-secondary binding partner complex, and which must be separately prepared for each first antibody. This imposes a preference for a first antibody of defined composition, for example a monoclonal antibody.

Summary of the Invention

According to the present invention, an assay for an analyte in a fluid sample comprises:

adding to the sample an excess of a labelled reagent which binds to the analyte, thereby forming a conjugate if analyte is present;

causing the sample to flow along a path that includes, immobilised at sequential first and second positions, a first species that binds the unconjugated reagent and a second species that binds the conjugate; and

determining bound conjugate.

For example, the method provided by the present invention requires that the solution which may contain analyte is mixed with an excess of a labelled antibody or other primary binding reagent. The mixture is allowed to react whilst migrating along a membrane or channel upon which firstly an analogue of the analyte and secondly a binder for the antibody or other primary binding reagent have been immobilised. Upon

contacting the analyte analogue, unused binding reagent is removed from the migrating mixture. Binding reagent-analyte conjugate is not so removed, and continues to migrate along the membrane or channel until it contacts the "test site", where it is concentrated for quantitation. The method of quantitation depends on the label employed; a preferred embodiment is visual or absorptiometric quantitation of coloured polystyrene microspheres.

The signal at the "test site" is higher for a greater amount of analyte. Excess labelled binding reagent is used over the amount of hapten, and also excess immobilised analogue and test-site binder over the amount of labelled binding reagent. Therefore, the signal obtained is relatively unaffected by the exact amount of reagents present.

The binding reagent is preferably an antibody or antibody fragment. In this context, and by contrast to the prior art, there is no requirement to prepare specific antibodies directed against the antibody-analyte complex; indeed, the capture reagent can be species-specific and obtained commercially. The method is equally applicable to a monoclonal, polyclonal or recombinant first antibody. Once the antibody has been prepared and labelled by conventional means, the only other reagent required individually for each analyte is a pure sample of the hapten of interest or its analogue, derivatised in such a way that it can be immobilised on the assay surface. A preferred method of achieving this is by coupling it to a protein which can adsorb to, say, nitrocellulose; ideally, this is the protein which was used for immunisation if a polyclonal antiserum containing antibodies against this protein is used.

Description of the Drawings

Figs. 1A, 1B and 1C are each schematic plan views of a device for use in the invention, respectively before, after (negative result) and after (positive result) use.

Fig. 2 shows the dose-response curve obtained by assaying samples containing various concentrations of atrazine, according to Example 2.

Description of the Invention

A suitable device for use in an assay of the present invention comprises a permeable material, or a channel or channels within an impermeable material, capable of transporting an aqueous solution. Such a material is shown in Fig. 1, and comprises an application zone 1, a first binding species immobilised on lines 2 transverse to the direction of flow indicated by the arrow, and a second binding species immobilised at a

test site 3. Such a device, and other features of the invention described herein, are for illustration only.

The device described above, together with a first reservoir from which the sample and labelled antibody are introduced and a second reservoir into which the reagents flow when they reach the end of the device, can be encased in an opaque housing. The test site may be viewed through a window in the housing. In the case of a multiple-line test site, only those lines selected by the designer as being suitable for quantitation or quality control are visible to the user through the transparent window.

The solution to be analysed is caused to flow through the device by a mechanism such as capillary action, non-bibulous flow or outside pressure. Labelled specific binding partners directed against the binding site on the analyte of interest are introduced at the application zone 1, so as to flow together with the solution carrying the analyte. These may be introduced by various means such as the rupturing of a separate reservoir, by separate addition, or by being included in or on the permeable material or channel(s). Any of the standard labels used for assays are suitable; examples are coloured particles, metal sols, coloured or fluorescent dyes and enzymes. Sufficient excess of the labelled specific binding partner is present to ensure that virtually all the analyte is bound by the specific binding partner, as is normal practice in a "sandwich" assay.

At a defined distance along the membrane or channel, e.g. in lines 2, the analyte or an analogue is immobilised. This may be bound to and displayed upon the surface of a protein. Sufficient of the immobilised species is used, to capture all of the labelled specific binding partner which has not previously bound the analyte of interest. The excess specific binding partner thereby becomes immobilised. Also in this region, if necessary or desired, there is immobilised a sample of the immunising carrier or such other material which may be required to remove contaminating antibodies with specificities other than for the analyte.

Labelled specific binding partner-analyte complexes are left to continue to migrate along the device. At the test-site 3, located at a defined distance along the membrane or channel, there is immobilised a second binding reagent with high affinity for the labelled specific binding partner-analyte conjugate, upon which this conjugate becomes concentrated.

In cases where the analyte is an antigen and the first binding partner is a labelled antibody, the immobilised binding partner may be, for example, a species-specific antibody directed against the first antibody, or a bacterial or recombinant protein such as Protein A, Protein G, or Protein L, or a hybrid thereof. In this case, and also in the general case, the first binding partner may, as well as being labelled as above, be derivatised in such as a way as to become part of a high-affinity binding pair, in which case the other partner of the high-affinity binding pair is used as the "test site". As an example of this, the first binding partner is biotinylated, and streptavidin or avidin is immobilised at the "test site".

The amount of label accumulated at the "test site" is controlled by the amount of analyte introduced to the device in the sample; the greater the amount of analyte, the higher the signal. By appropriate choice of binding partners, a threshold reaction can be produced in which a positive line is obtained only when the analyte sample is above a certain concentration. Alternatively, the intensity of the test line can be used to quantitate the level of analyte. The intensity of the line may be read visually or absorptiometrically in the case of a coloured label, or by whatever means is appropriate to the label if an alternative such as fluorescence is employed.

A smaller quantity of a "control" antibody may be used, such as an antibody or other protein which has no affinity for the analyte or the immobilised analyte analogue but which is bound with high affinity by the test site species and which is labelled differently from the assay antibody, or which is bound only by a control species located beyond the test site and which is labelled similarly to or differently from the assay antibody. If the sample does not contain any analyte, so that no signal is obtained, a control signal will be provided at the test site or at the control site to demonstrate the integrity of the reagents and their flow. In a preferred embodiment, this control antibody is labelled with a different colour of polystyrene microspheres whose accumulation may be determined visually. Another preferred embodiment utilises polystyrene microspheres of the same colour as those labelling the assay antibody but utilising a different chemistry for reaction, at the "control line", such as biotinylated polystyrene microspheres and a control line comprising immobilised streptavidin.

Another preferred feature of this invention comprises adding to the sample a labelled partner of an independent binding pair which does not interact with the analyte,

the reagent, the conjugate or either species, and wherein the flow path comprises, immobilised at a third position, the other partner of the independent binding pair. By determining the bound independent binding pair at the third position, this provides an indication of flow.

It will be clear to those skilled in the art that the affinity of the antibody, or other primary binding partner, for analyte in solution must be high and the affinity of antibody, or other primary binding partner, for the immobilised analyte or its analogue should be slightly less high. The immobilised second binding partner must also have high affinity for the labelled antibody or other primary binding partner and for any antibody used as a control.

The invention may be used to assay for any analyte for which there is a specific binding partner, for example a monoclonal, polyclonal or recombinant antibody, a receptor or a specific binding protein. It should be understood that the term "antibody" includes both complete immunoglobulin molecules and immunologically active fragments thereof. Examples of analytes include medicaments, drugs of abuse, agrochemicals, hormones, vitamins, peptides and protein. The analyte preferably has a molecular weight below 1000 Daltons. The invention is particularly suitable for assaying those molecular species for which it is advantageous to perform quantitation rapidly and where little technical expertise or instrumentation is needed or available.

The following Examples illustrate the invention.

Example 1 Detection of C Reactive Protein (CRP)

Strips of nitrocellulose (5 mm x 70 mm) were prepared as generally indicated by Fig. 1A. At the test site, 10 µg staphylococcal Protein A was immobilised as a narrow band across the width of the strip and, at a second site, 10 µg purified human CRP was immobilised as several such narrow bands in close proximity to each other. When fully dried, the strips were blocked with a buffer containing polyvinyl alcohol. Aqueous samples with and without CRP were mixed with an excess of a high-affinity antibody to CRP, coupled to coloured polystyrene microspheres. The mixture was allowed to migrate up the strips so that it passed over the immobilised CRP before coming into contact with the test line upon which CRP antibody-polystyrene became immobilised, giving a visible signal. In the absence of CRP, the particles became entrapped on the immobilised CRP and so did not reach the test line; in this case, no signal was seen there.

Example 2 Quantitation of atrazine (FW =216)

Strips of nitrocellulose (5 mm x 70 mm), nominal pore size 8μm, were prepared as generally indicated by Fig. 1A. At the test site, a stripe containing 6.25 μg recombinant protein G, and approximately 1cm distant from that a stripe containing 7.5 μg of an atrazine analogue conjugated to β-casein, were immobilised. The atrazine analogue used was not identical to that used to raise the antiserum. After drying, the nitrocellulose strips were blocked by saturation with a 0.025% solution of polyvinyl alcohol.

60 μl of an aqueous solution of atrazine in the μ/L concentration range was mixed with 2 μl of a suspension of sheep anti-atrazine immunoglobulin coupled to coloured polystyrene particles, and the whole was buffered to pH = 7.4. This was immediately allowed to travel along the nitrocellulose strip so that it reached the atrazine analogue before contacting Protein G at the test site. The immobilised atrazine analogue reacted with and removed from solution the excess antibody conjugate which had not reacted with atrazine analyte, but its lower binding affinity with the antiserum minimised reversal of the reaction between antibody and atrazine analyte.

The amount of light absorbed by the coloured particles accumulating at the protein G "test line" was recorded as a dose-response curve. In the absence of atrazine, there was no signal at the test-line; increasing atrazine content of the sample resulted in increasing signal. See Fig. 2, where signal intensity (SI; arbitrary units) is plotted against atrazine concentration ([A]; μg/L).

CLAIMS

1. An assay for an analyte in a fluid sample, which comprises:

adding to the sample an excess of a labelled reagent which binds to the analyte, thereby forming a conjugate if analyte is present;

causing the sample to flow along a path that includes, immobilised at sequential first and second positions, a first species that binds the unconjugated reagent and a second species that binds the conjugate; and

determining bound conjugate.

2. The method of claim 1, wherein the reagent is attached to one member of a high-affinity binding pair as well as to a label, and the second species is the other member of that pair.

3. The method of claim 2, wherein the said one member is biotin and the second species is avidin or streptavidin.

4. The method of any preceding claim, wherein the affinity of the reagent for the immobilised first species is less than its affinity for the analyte in solution.

5. The method of any preceding claim, wherein the first or second position comprises multiple lines or spots of the respective immobilised species, only some of which may be visible to the reader.

6. The method of any preceding claim, wherein the first position comprises multiple lines, transverse to the flow path, of the first species.

7. The method of any preceding claim, wherein the second position comprises one or more lines, transverse to the flow path, of the second species.

8. The method of any preceding claim, which additionally comprises adding to the sample an antibody, labelled differently from the reagent, and which binds the second species but not the first, thereby providing quality control in the event of a negative result.

9. The method of any preceding claim, which additionally comprises adding to the sample a labelled partner of an independent binding pair which does not interact with the analyte, the reagent, the conjugate or either species, and wherein the flow path comprises, immobilised at a third position, the other partner of the independent binding pair, and determining the bound independent binding pair at the third position, thereby providing an indication of flow.

10. The method of any preceding claim, wherein the analyte contains only one antigenic site.

11. The method of any preceding claim, wherein the flow path comprises one or more channels or permeable materials, in which liquid can flow via capillarity or wicking.

12. The method of any preceding claims, wherein the or each label is a solid particulate label, a fluorescent label or an enzyme.

13. The method of any preceding claim, wherein one or more of the first species, second species and (if present) said other partner of the independent binding pair, are immobilised on beads.

14. The method of any preceding claim, wherein the first species is the analyte or an analogue thereof.

15. The method of any preceding claim, wherein the analyte has a molecular weight of less than 1 kD.

16. The method of any preceding claim, wherein the affinity of the reagent for the analyte, the relative proportions of the reagent and the analyte, and the flow characteristics, are such that the conjugation reaction is complete, or close to completion, as the sample approaches the first position.

Fig.1A. Fig.1B. Fig.1C.

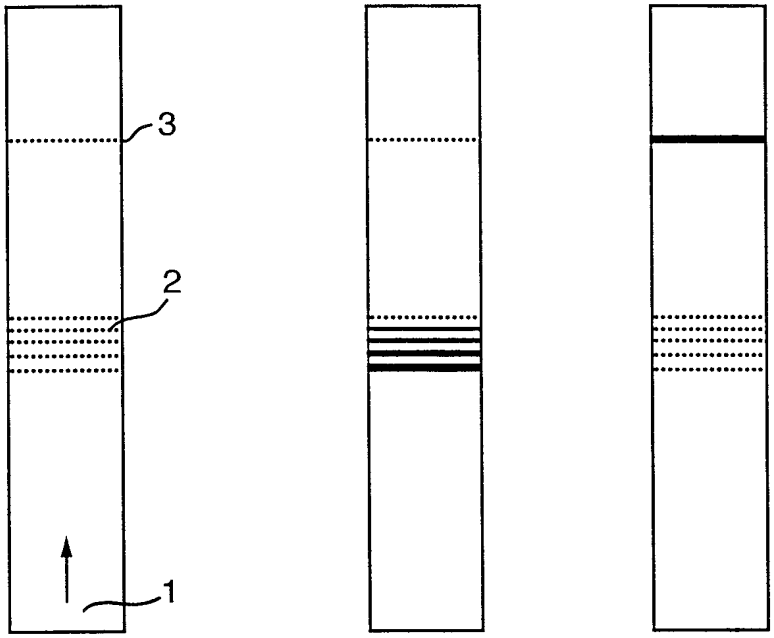
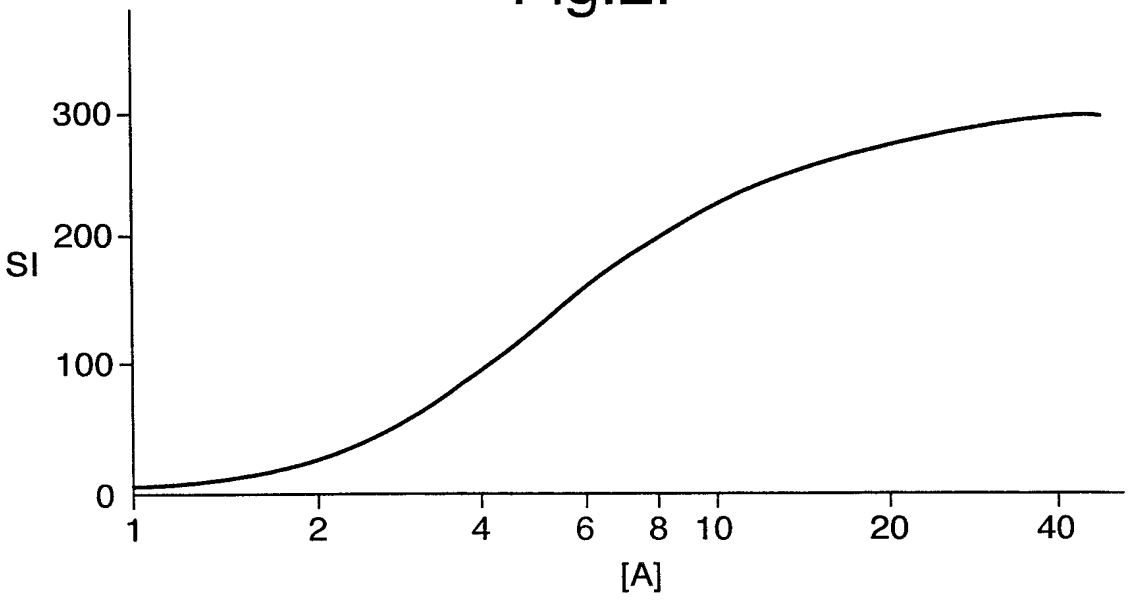


Fig.2.



INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/543 G01N33/558 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2 198 948 A (BAYER CORPORATION) 30 November 1997 (1997-11-30) the whole document	1-6
A	WO 97 34150 A (ABBOTT LAB) 18 September 1997 (1997-09-18) the whole document	1
P,A	WO 98 39657 A (QUIDEL CORP ;ROWLEY GERALD (US); BOEHRINGER HANS (US); PRONOVOST A) 11 September 1998 (1998-09-11) examples	1
P,A	EP 0 895 084 A (BAYER AG) 3 February 1999 (1999-02-03) examples	1

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☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
25 October 1999	04/11/1999

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Information on patent family members

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